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(71) Applicant *(for all designated States except US):* DIA-TECH LIMITED [GB/GB]; Egginton House, 25-28 Buckingham Gate, London SW1E 6LD (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : WILSON, Thomas, Michael, Aubrey [GB/GB]; Brookside, Barford Road, Marlingford, Norwich (GB).

(74) Agent: ELKINGTON AND FIFE; High Holborn House, 52/54 High Holborn, London WC1V 6SH (GB).

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(57) Abstract

A chimaeric RNA comprises the origin of assembly sequence of a helical rod-shaped virus such as tobacco mosaic virus together with at least one sequence coding for a foreign protein. The chimaeric RNA can be produced by producing cloned cDNA copies of the RNA origin of assembly sequence and cloned DNA sequences coding for a foreign protein, ligating the cloned DNA sequences in the correct orientation and transcribing the recombinant DNA in a suitable transcription vector system. When the chimaeric RNA is assembled in a preparation of the coat protein of the virus whose ri-gin of assembly sequence is included in the chimaeric RNA, a pseudovirus particle is produced in which the chimaeric RNA is encapsidated by the virus coat protein. The pseudovirus particle can be used as a general means for protecting the RNA or as a vector for expression of the RNA in a wide variety of hosts.

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Recombinant - RNA Packaging System

The present invention relates to a recombinant - RNA packaging system based on helical, rod-shaped viruses.

Helical, rod-shaped ribonucleocapsids, in particular plant viruses, consist of a hollow cylinder of viral coat protein encapsidating an RNA molecule. The assembly of certain plant viruses, particularly tobacco mosaic virus ("TMV"), has been studied quite extensively and it is known that *in vitro* TMV can be assembled from its constituent RNA molecule and coat protein in a spontaneous reaction. This reaction involves specific initiation at a nucleation site on the TMV RNA which is referred to as the origin of assembly sequence. Local sequence variations in the RNA, other than in the origin of assembly sequence, do not substantially affect the efficiency of assembly of the virus. It may be assumed that assembly of other helical, rod-shaped viruses is initiated and proceeds in a similar way.

It has now been found that a recombinant RNA can be produced which includes the origin of assembly sequence of a helical, rod-shaped virus together with RNA coding for a foreign protein (referred to herein as a chimaeric RNA), and that such a chimaeric RNA can be encapsidated by a preparation of the virus coat protein to form a virus-like particle (referred to herein as a pseudovirus particle). Such

pseudovirus particl s which include packaged RNA in a ribonuclease resistant form provide general vehicles for the protection, delivery and expression of said RNA.

The present invention provides a chimaeric RNA comprising the origin of assembly sequence of a helical, rod-shaped plant virus together with at least one sequence coding for a foreign protein.

The invention also provides a process for preparing a chimaeric RNA comprising the origin of assembly sequence of a helical, rod-shaped plant virus together with at least one sequence coding for a foreign protein, which comprises producing cloned cDNA copies of the RNA origin of assembly sequence and at least one cloned DNA sequence coding for a foreign protein, ligating the cloned DNA sequences in the correct orientation and transcribing the recombinant DNA in a suitable transcription vector system to produce the chimaeric RNA.

The invention also provides a pseudovirus particle comprising a chimaeric RNA which comprises the origin of assembly sequence of a helical rod-shaped plant virus together with at least one sequence coding for a foreign protein, encapsidated by the coat protein of the virus whose origin of assembly sequence is included in the chimaeric RNA.

The invention further provides a process for the production of a pseudovirus particle which comprises assembly of a chimaeric RNA comprising the origin of assembly sequence

of a helical, rod-shap d plant virus togeth r with at least ne sequence coding for a foreign protein in a preparation of the coat protein of the virus whose origin of assembly sequence is included in the chimaeric RNA.

Finally the invention provides a method for the expression of a heterologous protein in a host which comprises pseudo-infecting the said host with a pseudovirus particle comprising a chimaeric RNA which comprises the origin of assembly sequence of a helical rod-shaped plant virus together with a sequence coding for the foreign protein encapsidated by a coat protein of the virus whose origin of assembly sequence is included in the chimaeric RNA.

The pseudovirus particle according to the invention represents a vector which can be directly synthesised in large amounts and which contains RNA in a packaged form. Accordingly the pseudovirus particles can be used as a general means for the handling and storage of otherwise labile _in vitro_ RNA transcripts.

The pseudovirus particles can be introduced into and expressed in a wide variety of hosts which extends far beyond the normal host for the virus on which the pseudovirus particle is based. In the case of pseudovirus particles derived from a plant virus such as TMV, the pseudovirus particle represents an expression vector which, because of its packaging, is stable in the extra-cellular state and which is capable of "pseudo-infection" of plant cells which have not

been subjected to any special treatment, i.e. the intact plant, as opposed to protoplasts, callus or suspension cultured cells.

It is now believed that host specificity of plant viruses does not reside at the level of virus uptake into the cells which appears to be completely non-specific. Disassembly and early translation events also seem to occur in a wide range of host and non-host plants alike and true host specificity lies only in the availability or identity of certain components of the viral RNA replicase complex or cell-to-cell spread. Expression of pseudovirus particles derived from TMV has been observed in a plant species which is classified as a poor "subliminal" host for TMV. Accordingly the pseudovirus particles according to the invention based on plant viruses can be used as transient expression vectors in a wide range of hosts beyond the normal host of the plant virus concerned.

It has also beeen demonstrated that animal cells (Xenopus laevis oocytes) are capable of uncoating pseudovirus particles according to the invention and expressing the encapsidated mRNA. Uncoating and expression is also possible in a cell free system derived from Escherichia coli cells. Accordingly the range of suitable hosts for pseudovirus particles according to the invention based on helical rod-shaped plant viruses appears very wide indeed and is not confined to plant cells

In principle the invention can be applied to any helical,

rod-shaped plant virus which assembles under the control of an origin of assembly sequence in a manner which is independent of the length and sequence of the remainder of the RNA. As noted above TMV has been extensively characterised, including the origin of assembly sequence. However the invention can also be applied to other helical, rod-shaped plant viruses.

Suitable plant viruses apart from TMV include potato virus X which has the advantage that useful amounts of free coat protein for in vitro assembly of recombinant RNA transcripts are available in a workable form. However present evidence suggests that the major mono-directional assembly mechanism is 5'→3' and would require the potato virus X origin of assembly sequence upstream of the RNA sequence coding for the foreign protein of interest.

As used herein the term "origin of assembly sequence" of a helical rod-shaped plant virus means that part of the RNA sequence of the virus which is essential for a chimaeric RNA to be assembled into pseudovirus particles in the presence of the appropriate coat protein. A 126 nucleotide sequence located between residues 5420 and 5546 from the 5' end of the TMV RNA molecule has been identified as the core of the sequence required for the nucleation of the TMV assembly with the residues 5313 to 5546 (the so-called extended region) also being implicated (see Goelet et al, Proc. Nat. Acad. Sci. U.S.A. 79, 5818-5822 (1982) and Zimmern et al, Cell, 11 455-462 (1977)), although not all of this sequence is essential to

effect assembly (Turner & Butler, Nuc. Acids Res, 14 9229 (1986)).

It is possible to prepare chimaeric RNA by directly ligating the TMV origin of assembly sequence to an RNA coding for a foreign protein using T4 RNA ligase. However this process is of low efficiency.

It is preferred to produce cloned cDNA copies of the RNA origin of assembly sequence and of the cloned DNA sequences coding for a foreign protein, ligate the cloned DNA copies and transcribe the recombinant DNA in a suitable transcription system. The sequence coding for the foreign protein is also used in the form of DNA and both DNAs are inserted in a suitable orientation into a transcription vector.

Suitable vectors include the SP6 RNA polymerase plasmids pSP64 and pSP65 which are commercially available (Promega Biotec, Madison, WI, USA) and which contain a strong promoter for bacteriophage SP6 RNA polymerase.

Other suitable plasmids include the dual promoter plasmids (e.g. pGEM 1-4 also available from Promega Biotec) which use SP6, T3 and/or T7 RNA polymerases. Thus, by orienting an origin of assembly sequence in an assembly-competent manner at both ends of the intervening M13 poly-linker sequence, any central foreign insert can be run off and packaged 3'$\longrightarrow$5' in either the positive or negative (anti)-sense. Addition of rho-independent transcription termination signals would probably enhance the overall yield of

transcripts if arranged outside the motif described above since the template would no longer need to be linearized. Transcripts can be 5'-capped, by using, for example, m^7Gppp... as a primer for transcription, to prolong and enhance their cellular life and activity.

In vitro packaging of the chimaeric RNA transcripts can be carried out using a prefabricated "disk" preparation of TMV coat protein under the assembly conditions published by Butler, J. Gen. Virol. 65 253-279 (1984) and Durham, J. Mol. Biol. 67 289-305 (1972). Assembly can be monitored turbidometrically at 310 nm using unlabelled transcripts, by recovery of micrococcal nuclease-resistant ^{32}P-labelled transcripts or by electron microscopy of negatively-stained nucleoprotein helices.

The original pseudovirus particles described above are essentially single round expression vector systems in the sense that they are not designed to replicate in "pseudo-infected" plants. On this basis their host range should be wide, including monocotyledons, since host range probably does not rely on specificity of early uptake, uncoating and primary gene expression events. A pseudovirus particle based on TMV as described above should thus be capable of expression in any plant cell and as noted above the host range extends beyond plant cells. It may be possible to prepare pseudovirus particles according to the invention which are capable of replication but these will probably be more host-specific.

The essential el ments of th vectors according to the invention (viral origin of assembly sequence plus available cognate coat protein) can be used in conjunction with any other putative "vector/delivery" system to provide protective packaging for RNA constructs which are larger than otherwise tolerable in, for example, an isometric (spherical) nucleocapsid.

Assembly of chimaeric RNA into pseudovirus particles is illustrated by the following Examples 1 to 5 and expression of the chimaeric RNA is illustrated in Examples 6 to 8. In the examples reference is made to the following Figures:

Figure 1 represents pSP64-derived constructs capable of directing the synthesis of specific RNA's containing the TMV origin of assembly sequence.

Figure 2 illustrates electrophoresis of linearized pSP64-derived RNA constructs before and after encapsidation, or following exposure to ribonuclease.

Figure 3 shows the results of electron microscopy of packaged in vitro transcripts.

Figure 4 shows the results of sucrose-density gradient fractionation of packaged, labelled SP6-transcripts.

Recombinant plasmids designated pSP64TMV, pSP64CT, pSP64LT, pSP64LRT, pJII1 and pJII2 which contain the TMV origin of assembly sequence (OAS) were constructed using the commercially available pSP64 plasmid (Promega Biotec) which contains the strong promoter for bacteriophage SP6 RNA

polym rase. All plasmid constructs were grown in Escherichia coli strain DH1 and prepared using standard procedures as described by Maniatis, T., Fritsch, E.F., and Sambrook., J. in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1982).

The pSP64-derived plasmids are illustrated in Figure 1 in which the arrows represent the start of transcription by SP6 RNA polymerase.

Example 1

(A). Plasmid pSP64TMV containing a 440 bp cDNA sequence including the TMV OAS.

A fragment corresponding to residues 5118-5550 of the total TMV (vulgare strain) RNA sequence and containing both the "core" (positions 5420-5546) and extended (position 5313-5546) OAS region was supplied by Dr. P. Goelet as an MspI fragment of TMV cDNA inserted into the AccI site of M13mp7 (Goelet and Karn, Gene 29, 331-342 (1984)). The TMV OAS was excised as a BamHI fragment and subcloned into M13mp10 (commercially available from New England Biolabs, see Messing, Methods in Enzymology, 101, 20 (1983)) from which a clone containing the OAS, in the desired orientation for later 3'⟶5' assembly, was selected by sequencing using the method of Sanger et al ., J. Mol. Biol. 143 161-178 (1980). The TMV fragment was then excised from M13mp10 by double-digestion with EcoRI and SalI and cloned into pSP64 to produce pSP64TMV.

When linearized with EcoRI, this construct produces transcripts of 508 nucleotides (n).

(B). <u>Plasmid pSP64CT containing a cDNA sequence coding for calf preprochymosin in addition to the TMV OAS</u>.

A cDNA sequence coding for calf preprochymosin (see Harris <u>et al</u>, Nucleic Acid. Res. <u>10</u> 2177-2187 (1982)) was supplied as plasmid pSP64Chy82^{+} by Dr. A. Colman (University of Warwick) as a 1151 bp BclI fragment with HindIII linkers, inserted into the HindIII site of pSP64 (see Drummond <u>et al</u>, Nucleic Acid Res. <u>13</u> 7375-7394 where the plasmid pSP64 Chy82^{+} is referred to on page 7377 as psp82^{+}). This HindIII fragment was transferred directly into the HindIII site of pSP64TMV to produce pSP64CT. The orientation was determined by restriction mapping. When linearized with SacI, this construct produces transcripts of 1659n, containing a 3'-OAS.

(C). <u>Plasmid pSP64LT containing a cDNA sequence coding for chicken lysozyme in addition to the TMV OAS</u>.

A 485 bp HindIII-linkered cDNA including the entire coding region for chicken lysozyme (see Jung <u>et al</u>, Proc. Nat. Acad. Sci. U.S.A. <u>77</u>, 5759-5763 (1980)) was provided in pUC8Lys^{+} by Dr. A. Colman (University of Warwick). pUC8Lys^{+} is a HindIII-linkered fragment from pTK$_2$Lys^{+} (see Drummond <u>et</u>

al., Nucleic Acids Res. 13 7375-7394 (1985), which contains the chicken lysozyme gene which was inserted into pUC8 (commercially available from Bethesda Research Labs (see Vieira et al., Gene 19, 259 (1982)). This fragment was transferred to the HindIII site of pSP64TMV to produce pSP64LT. When linearized with EcoRI, this construct produces transcripts of 993n and when linearized with BamHI it produces transcripts of 523n, lacking the TMV OAS.

(D). Plasmid pSP64LRT - identical to pSP64LT except for the addition of a 5.3kbp rDNA fragment between the lysozyme coding region and the TMV OAS.

A 5.3kbp EcoRI fragment of *Xenopus borealis* rDNA containing the 18S, ITS-1, 5.8S, ITS-2 and 28S sequences was excised from pXbr101, provided by Prof. B.E.H. Maden (University of Liverpool) (see Furlong & Maden, EMBO J. 2, 443-448 (1983)). The EcoRI fragment was blunt-ended and cloned into the HindIII site of pSP64TMV to produce pSP64RT. Insert orientation was determined by restriction mapping. The HindIII fragment containing the lysozyme cDNA sequence was inserted into the HindIII site of pSP64RT, as described above for pSP64LT, to produce pSP64LRT. When linearized with EcoRI, this construct will produce transcripts of 6250n. This plasmid was designed to produce long transcripts (6.25kb) for packaging studies, together with the 5'-open reading frame encoding an immunodetectable polypeptide alien to plant cells.

(E). <u>Plasmid pJII2 containing a cDNA sequence coding for chloramphenicol acetyl transferase in addition to the TMV OAS</u>.

The plasmid pJII1 is derived from pSP64TMV by linker conversion, wherein pSP64TMV is cut with *Sma*I and ligated with *Bgl*II (commercially available) linkers. A 779 bp *Sal*I fragment of pCM1 (Close, T.J. *et al*., Gene, 20, 305-316 (1982)) containing the chloroamphenicol acetyl transferase (CAT) gene of Tn9 was introduced into the *Sal*I site of pJII1 to give pJII2.

<u>Example 2</u>

<u>Assembly and protection of *in vitro* transcripts.</u>

^{32}P-rUTP-labelled SP6 transcripts were synthesised as described by Melton *et al*., Nucleic Acid Res. 12, 7035-7056 (1984) and Butler *et al*., J. Biol. Chem. 257 5779-5788 (1982), in a total reaction volume of 50 microlitres, containing 1 microgram linearized plasmid DNA but lacking BSA. Transcripts were recovered, without DNase digestion, by phenol/chloroform extraction and ethanol precipitation. *In vitro* packaging reactions used a prefabricated "disk" preparation of TMV coat protein at an approximate protein:RNA ratio of 100:1 under published assembly conditions (see Butler, J. Gen. Virol. 65, 253-279 (1984) and Durham, J. Mol. Biol. 67, 289-305 (1972)). Naked or packaged transcripts were incubated at 20^{o}C with 40U/ml micrococcal nuclease and 1mM $CaCl_2$. Reactions were

stopped by addition of EGTA to 10mM and SDS to 2% (w/v) prior to phenol/choloroform extraction and thanol precipitation.

The following transcripts were analyzed by electrophoresis on a 1% (w/v) agarose/7.5% formaldehyde/MOPS denaturing gel system (Kreig _et al._, Nucleic Acid. Res. _12_, 7057-7070 (1984)):

A, EcoRI-linearized pSP64TMV;

B, BamHI-linearized pSP64LT;

C, EcoRI-linearized pSP64LT;

D, SacI-linearized pSP64CT; and

E, EcoRI-linearized pSP64LRT.

The results are shown in Fig 2 and for each transcript, tracks 1-4 represent the initial SP6-transcript (1), the naked transcript digested with micrococcal nuclease (2), the transcript incubated with TMV protein for 1 hr at 20^{o}C and recovered without nuclease digestion (3) or following 30 min digestion with micrococcal nuclease (4).

Tracks marked M represent SP6 transcripts of known size (235n, 683n, 1442n or 1784n) produced by linearizing pSP65 at known restriction sites for PvuII, DdeI, SinI or ScaI respectively.

Example 3

Electron microscopy of in vitro transcripts negatively stained with uranyl acetate.

The results of electron microscopy are shown in Figure 3.

Samples of _in vitro_ assembly reactions containing TMV coat protein "disks" alone (panel A), or with unlabelled RNA transcripts from pSP64TMV (panel B), pSP64CT (panel C), or pSP64LRT (panel D) were viewed in a Philips EM400 and representative micrographs printed at a final magnification of 108,000X for direct measurement of nucleoprotein rodlet lengths. The histograms shown in panels E-G, correspond to material shown in panels B-D respectively. The heavy black bars represent 100nm.

Example 4

Sucrose density-gradient fractionation of packaged, labelled SP6 transcripts

pSP64LT was linearized with EcoRI or BamHI. The latter enzyme removed a DNA fragment corresponding to the TMV OAS sequence (compare Fig. 2., tracks B1 & C1). 1.5 microgram of either template was incubated under standard (50 microlitre) reaction conditions (see Example 2) with 100 micro molar unlabelled rUTP and 10 micro Curies alpha-[^{32}p]-rUTP for 2hr initially with 15U SP6 RNA polymerase (Boehringer, Mannheim). An additional 10U of polymerase were added after 1hr. Radiolabelled transcripts were recovered as described and two aliquots, each equivalent to 25% of the total yield of RNA, were packaged separately with TMV protein at an estimated protein:RNA ratio of 100:1. One sample was stored on ice

while the second was digested at $20^{\circ}C$ with micrococcal nucleas (Bo hring r, Mannheim) at 300U/ml in 3mM $CaCl_2$. After 30min, EGTA was added to 5mM final concentration. All samples (in 100 microlitres), including 12.5% aliquots of the original RNA transcripts, were loaded onto linear, 15-30% (w/v) DEP-treated sucrose density-gradients (5ml), buffered with 0.1M Tris-HCl, pH8.0 at $5^{\circ}C$. Gradients were spun at 45,000 rpm for 3 hrs in a Beckman SW50.1 rotor. Forty, 5-drop fractions were collected from each gradient and the Cerenkov radiation associated with each fraction was measured. The results are shown in Figure 4. Gradients 1-3 contained EcoRI-cut pSP64LT transcripts either alone (□---□), packaged (△---△), or packaged and nuclease-digested (○---○). Gradients 4-6 contained BamHI-cut pSP46LT transcripts either alone (□---□), "packaged" (△---△), or "packaged" and nuclease-digested (○---○). Sedimentation was from right to left in each case.

Discussion

Quantitative analysis of the nucleoprotein rods recovered from the <u>in vitro</u> packaging reactions resulted in the histograms shown in Fig. 3E-G. Based on the predicted (Fig. 1) and actual (Fig. 2, tracks A1, C1, D1, E1) lengths of the <u>in vitro</u> transcripts rods of 21nm, 40nm, 75nm or 290nm were expected to predominate following encapsidation of "competent" RNAs from pSP64TMV, pSP64LT, pSP64CT or pSP64LRT respectively.

This is approximately true for pSP64TMV-derived RNA (Fig.3 B,E), allowing for lik ly additional turns of protein subunits at either end of the rodlets, and also for EcoRI-cut pSP64LT-transcripts (EM data not shown, but see protected RNAs in Fig. 2, track C4). However, the results from pSP64CT or pSP64LRT transcripts are more complex. It is suggested that the 3'→5' stepwise assembly process proceeds for approx. 1.0-1.5kb along the 6.25 kb chimaeric pSP64LRT-transcript (Fig. 2, track E1) until some extensive, stable hairpin-loop structure, in the 28S rRNA portion, prevents further elongation by failing to melt and enter up the central hole of the growing nucleoprotein helix. Fig.3D confirms the presence of adequate amounts of free TMV protein to complete the assembly, while Fig.3G demonstrates the predominance of 70-90nm nucleoprotein rodlets. In the case of pSP64CT-derived RNAs, a significant fraction (about 25%) are fully-encapsidated [Fig.2, track D4 (visible on original autoradiograph)], while the majority appear to be only partially-coated to form 45-60nm rodlets (Fig.3C,F). The shorter, protected RNA (i.e. less than 1.6kb, but greater than OAS itself (0.44kb)] recovered in Fig. 2, track D4, probably represents this fraction of the packaged, pSP64CT-transcript population.

The efficiency of encapsidation of the chimaeric RNA transcripts can be estimated by (i) measuring the absolute concentration of rodlets observed in the electron microscope (Fig. 3), (ii) calculation from the yield of radiolabelled

transcripts recovered in nucleoprotein structures following micrococcal-nuclease digestion (Fig. 2, tracks A4-E4), or (iii) sucrose density-gradient ultra-centrifugation of the assembled, radiolabelled transcripts, as shown in Fig. 4. By the latter two methods, it was routinely found that approx. 40-60% of the input RNA was recovered in a stable packaged form. Variations in efficiency probably reflect the variable quality of the TMV coat protein "disk" preparations used.

Production of pseudovirus particles containing RNA coding for a readily assayable protein (CAT) and expression thereof is illustrated in the following examples 5 to 8. In these examples "buffer" refers to 0.25M Tris-HCl, pH 7.4, containing 10mM dithiothreitol and 2mM leupeptin.

Example 5

Pseudovirus particles were prepared from BglII linearized plasmid pJII2 by the method described in Example 2, except that the steps of ^{32}P-rUTP labelling and incubation of the naked or packaged transcripts at 20^{o}C with micrococcal nuclease and $CaCl_2$ were omitted. Pseudovirus particles were also prepared from capped transcripts of linearized pJII2 using standard techniques. The majority of pseudovirus particles produced corresponded to the predicted length for CAT pseudovirus particles of about 60nm.

Example 6

Tobacco cells are natural hosts for TMV. Tobacco

mesophyll protoplasts were polyethylene glycol inoculated (Dawson et al., Z. Naturforsch. C. Biosci. 33, 548 (1978)) with the following preparations

1) PEG alone
2) CAT mRNA
3) CAT pseudovirus particle
4) 5'- capped CAT mRNA
5) 5'- capped CAT pseudovirus particles.

Samples 2 and 4 represent the mRNA transcripts from the linearized plasmid pJII2 used to produce pseudovirus particles in accordance with Example 5 and samples 3 and 5 represent the pseudovirus particles themselves produced in accordance with Example 5. Samples 2 - 5 received equivalent amounts of RNA on a weight basis.

Following innoculation the protoplasts were incubated at 25°C for 20 hours. Protoplasts were removed from isotonic culture medium (Dawson et al supra) by centrifugation, then resuspended and sonicated (10 sec) in an equal volume of buffer. Cellular debris was removed by centrifugation at 10,000 x g for 10 minutes at 4°C and 100 microlitre samples of each supernatant were assayed for CAT activity, by the method of Gorman et al (Mol. Cell Biol. 2 1044 (1982)). 0.025 Units of purified CAT were added to the sample from the protoplasts inoculated with PEG alone as a reference.

The assay for CAT activity showed that the pseudovirus particles 3 and 5 produced a level of activity comparable to

or greater than that produced by the corresponding naked mRNA.

Example 7

Pea (Pisum sativum L) is classified as one of the poorest "subliminal" hosts for TMV (Cheo, P.C. and Gerard, J.S. Phytopathology 61, 1010 (1971)). CAT-expression in epidermal cells of Argenteum pea was investigated by inoculating pseudovirus particles or equivalent amounts of unencapsidated CAT mRNA constructs directly onto the leaf surface with silicon carbide [Carborundum 180 grit] as an abrasive. The mutant Argenteum (Marx, J. Heredity 73 413 (1982)) was used in view of its easily-peeled epidermis.

The preparations tested were as for Example 5 except that buffer was used as a control. Other samples were applied so that equivalent amounts of RNA on a weight basis were used.

Strips of epidermal cells were removed after 90 minutes and stored in liquid nitrogen (Shaw et al., Virology 148 326 (1986)) before being ground to a frozen powder. Lysed cells were resuspended in 300 microlitres of buffer. Cellular debris were removed and CAT assays performed as described in Example 5. 0.1 Unit of purified CAT was added to the sample inoculated with buffer as a reference.

The assay for CAT activity again showed that the pseudovirus particles 3 and 5 produced a level of activity comparable to or greater than that produced by the corresponding naked mRNA.

Example 8

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To determine whether pseudovirus particles could be uncoated and the mRNA expressed in animal cells Xenopus laevis oocytes were micro-injected separately with water as a control and with equivalent amounts of preparations 2 to 5 referred to in Example 5. Oocytes were also injected with the linearized plasmid DNA template containing the CAT coding sequence and the TMV origin of assembly to rule out any coupled transcription-translation activity. For further details of the methodology of oocyte injection see Colman in Transcription and Translation: A Practical Approach Ed. Hames et al IRL Press, Oxford (1984) pages 271-302.

After incubation at 20°C for 18 hours, equal numbers of viable oocytes were lysed and centrifuged at 10,000 x g for 5 minutes to sediment yolk material and float off lipids. The intervening liquid was removed in each case for CAT assay. 0.1 Unit purified CAT was added to a sample of the water-inoculated oocyte extract as a reference.

The assay for CAT activity showed that the pseudovirus particles 3 and 5 produced CAT activity in the Xenopus oocyte system.

Unlike tobacco or pea cells, the Xenopus oocyte system responded more efficiently to non-encapsidated CAT mRNAs than to the corresponding pseudovirus particles. However, a significant number of pseudovirus particles were disassembled and the resulting CAT mRNA expressed. This result suggests that the cytoplasm of animal cells includes suitable and

sufficient machinery to disassemble the pseudovirus particles according to the inventi n.

CLAIMS:

1. A chimaeric RNA comprising the origin of assembly sequence of a helical rod-shaped plant virus together with at least one sequence coding for a foreign protein.

2. A chimaeric RNA according to claim 1 wherein the plant virus is tobacco mosaic virus.

3. A process for preparing a chimaeric RNA according to claim 1 or 2 which comprises producing cloned cDNA copies of the RNA origin of assembly sequence and cloned DNA sequences coding for a foreign protein, ligating the cloned DNA sequences in the correct orientation and transcribing the recombinant DNA in a suitable transcription vector system to produce the chimaeric RNA.

4. A pseudovirus particle comprising a chimaeric RNA according to claims 1 or 2 encapsidated by the coat protein of the virus whose origin of assembly sequence is included in the chimaeric RNA.

5. A process for the production of a pseudovirus particle which comprises assembly of a chimaeric RNA according claim 1 or 2 in a preparation of the coat protein of the virus whose origin of assembly sequence is included in the chimaeric RNA.

6. A method for the expression of a heterologous protein in a host cell which comprises pseudo-infecting the said host cell with a pseudovirus particle according to claim 4 in which

the sequence coding for a foreign pr tein is a sequence coding for the heter logous protein.

7. A method according to claim 6 wherein the host cell is a plant cell.

8. A method according to claim 6 wherein the host cell is an animal cell.

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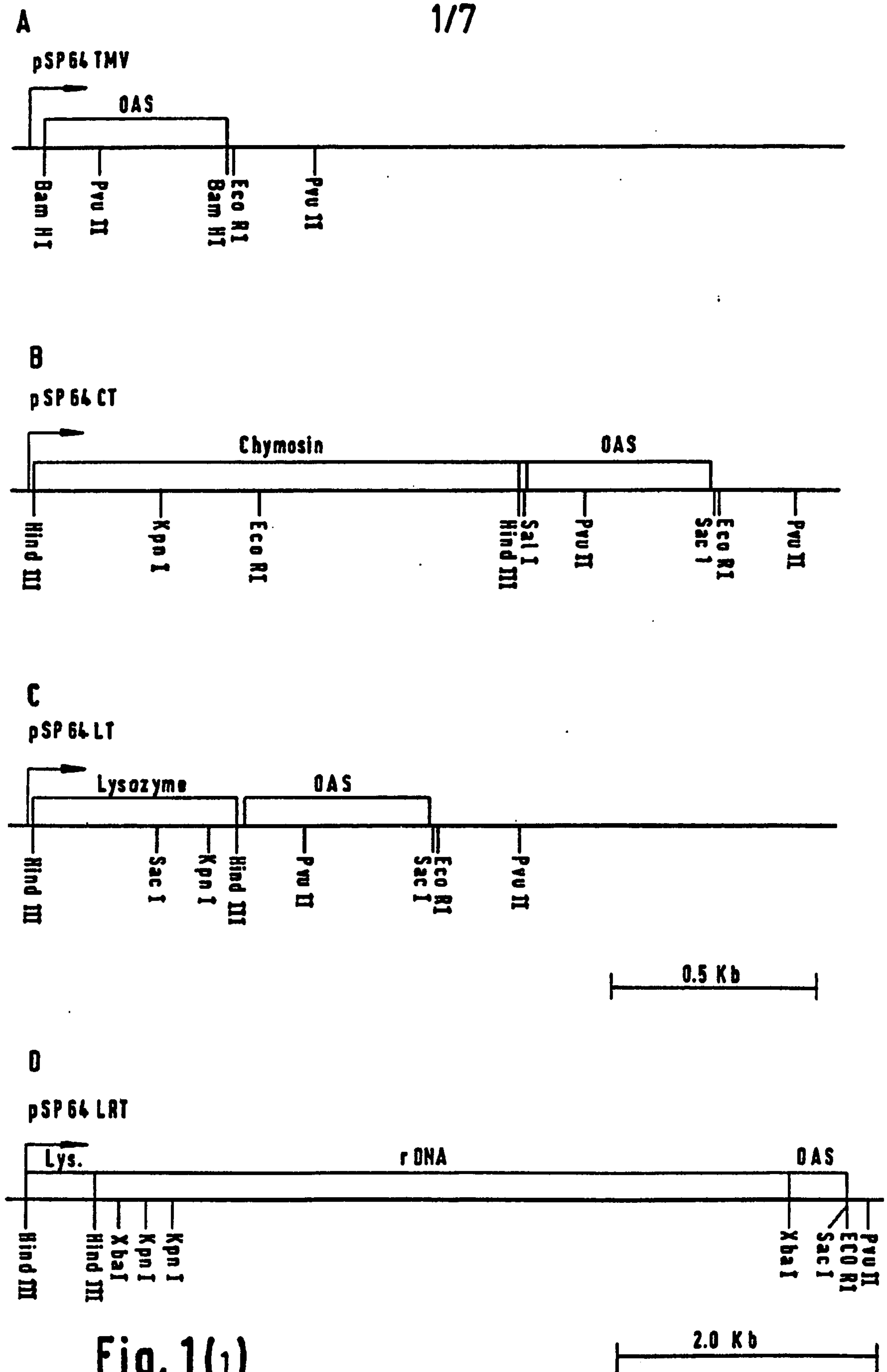

Fig. 1(1)

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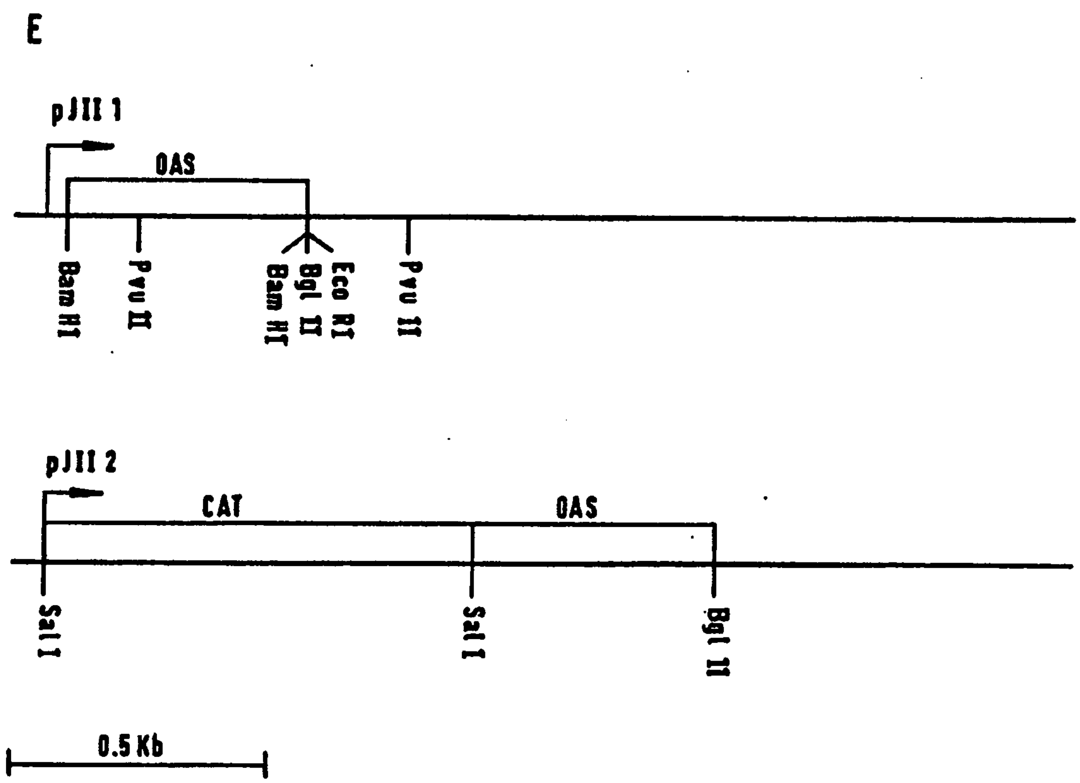
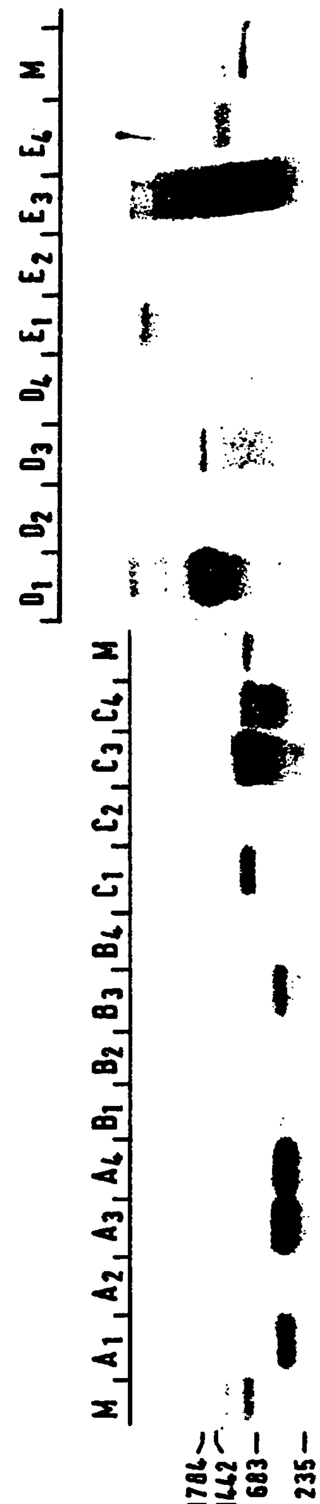


Fig. 1(2)

12/10/2001, EAST Version: 1.02.0008

3/7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E4
M
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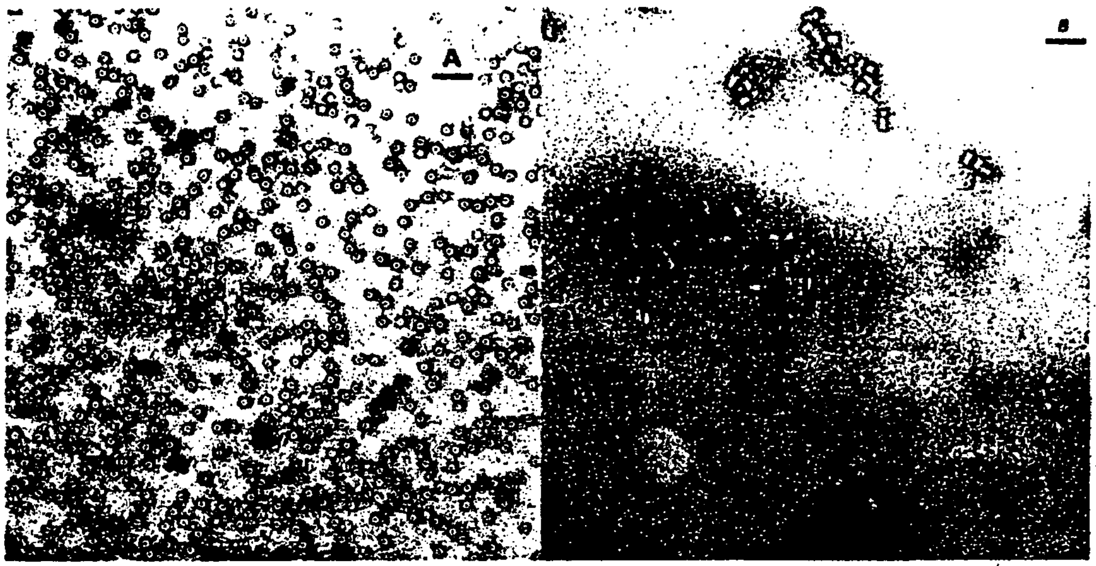
1442
683
235

Fig. 2



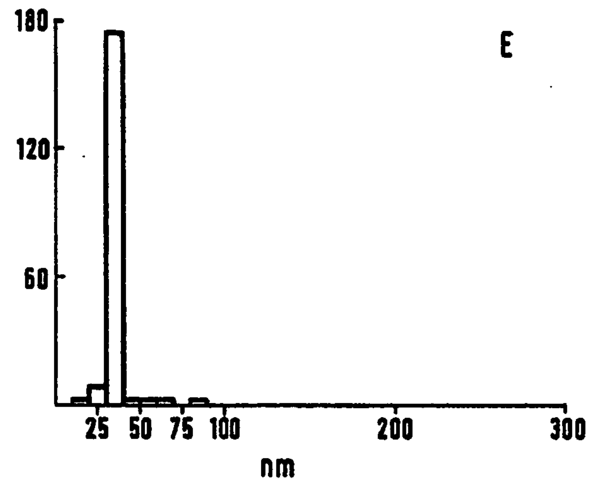


Fig. 3(1)

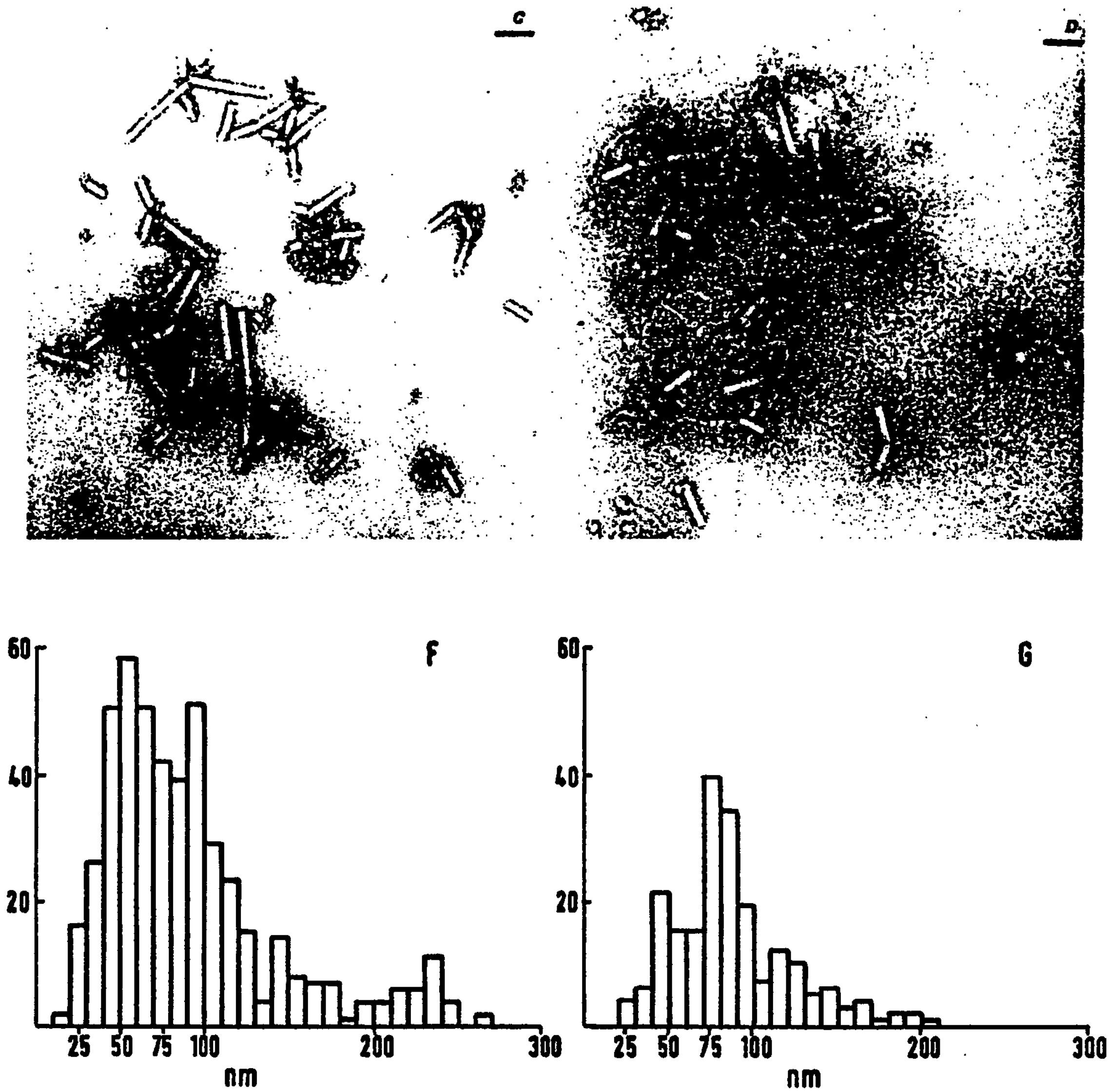


Fig. 3(2)

12/10/2001, EAST Version: 1.02.0008

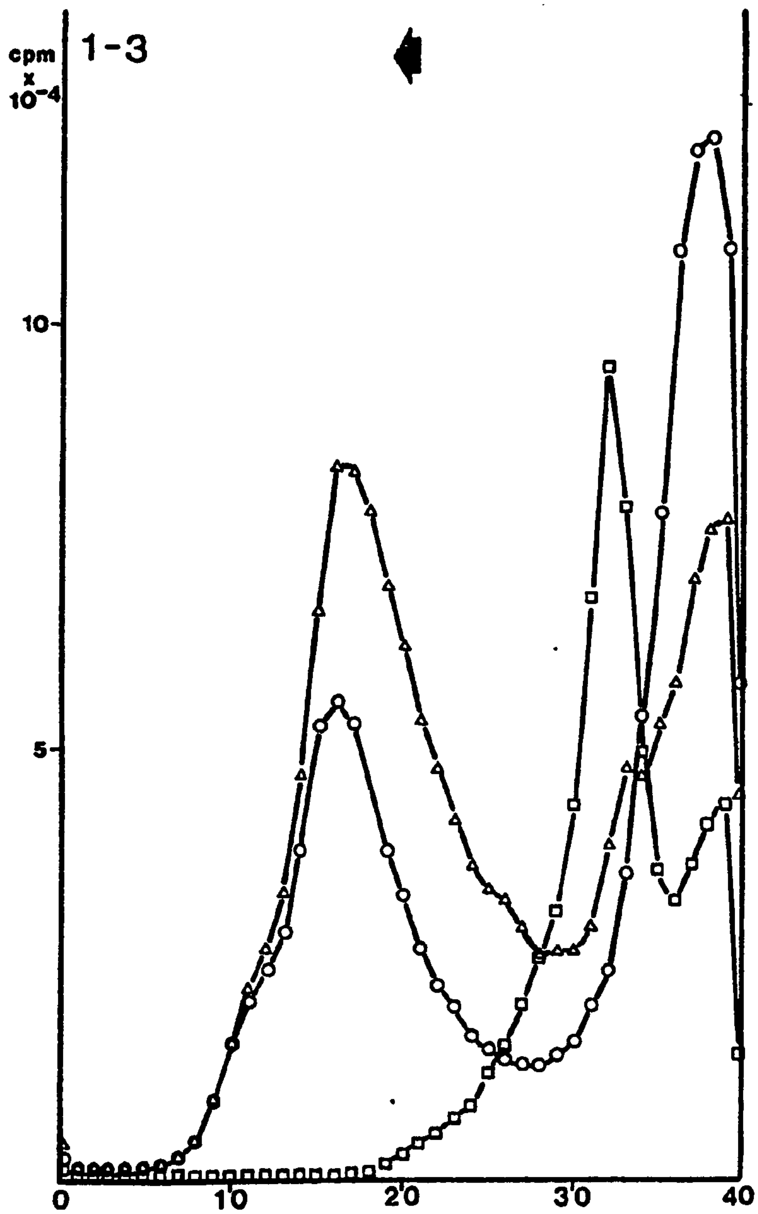


Fig. 4 (1)

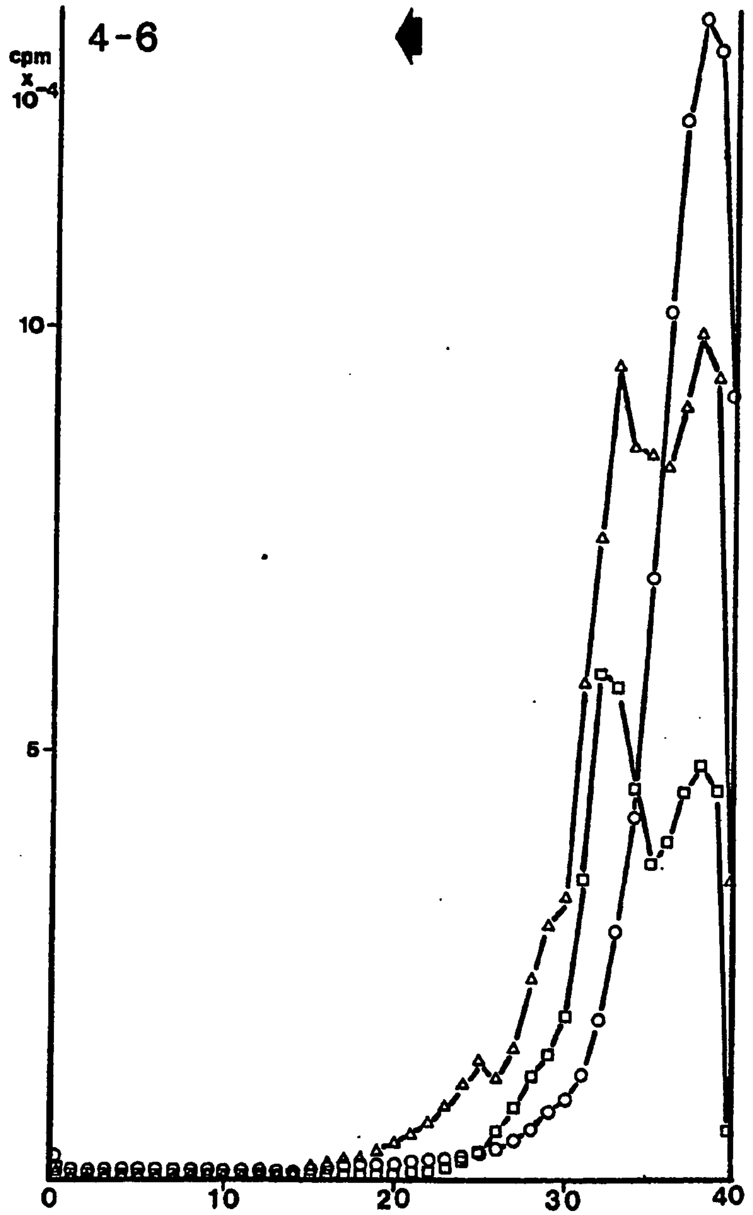


Fig. 4(2)

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 87/00249

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4: C 12 N 15/00; C 12 P 21/02; C 12 N 7/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC^4	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP, A, 0067553 (NATIONAL RESEARCH COUNCIL OF CANADA) 22 December 1982 see page 5, lines 8-11; page 5, lines 21-23; page 6, line 18 - page 7, line 4; page 9, lines 3-6; page 10, lines 5-11; page 15, lines 3-7; page 18, line 1 - page 21, line 7; page 22, line 20 - page 25, line 5; page 25, lines 24-32 --	1-7
X	Chemical Abstracts, volume 106, 1987, (Columbus, Ohio, US), D.E. Sleat et al.: "Packaging of recombinant RNA molecules into pseudovirus particles directed by the origin-of-assembly sequence from tobacco mosaic virus RNA", see page 158, abstract 62188h, Virology 1986, 155(2), 299-308 --	1-8
A	Proc. Natl. Acad. Sci. USA, volume 83, March 1986, W.O. Dawson et al.: "cDNA cloning of the complete genome of tobacco mosaic virus and production of ./.	

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23rd July 1987	21 AUG 1987
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	infectious transcripts", pages 1832-1836 see the abstract	1-8
A	Chemical Abstracts, volume 104, 1986, (Columbus, Ohio, US), M. Tabler et al.: "Infectivity studies on different potato spindle tuber viroid (PSTV) RNAs synthesized in vitro with the SP6 transcription system", see pages 137-138, abstract 29605c, EMBO J. 1985, 4(9), 2191-9	1-8
A	EP, A, 0153154 (AGRIGENETICS) 28 August 1985 see page 4, last paragraph - page 5, paragraph 4	1-8
P,X	EP, A, 0194809 (LUBRIZOL GENETICS) 17 September 1986 see claims 1,2,10,21,22,26,27,30	1,2,4,6-8
A	Chemical Abstracts, volume 80, 1974, (Columbus, Ohio, US), D.R. Black et al.: "Structure and infectivity of picornaviral RNA encapsidated by cowpea chlorotic mottle virus protein", see page 130, abstract 35047z, J. Virol. 1973, 12(6), 1209-15	1-8
A	Chemical Abstracts, volume 102, 1985, (Columbus, Ohio, US), D'Ann Rochon et al.: "TMV coat protein encapsidates specific species of host RNA both in vivo and in vitro", see page 341, abstract 163542s, UCLA Symp. Mol. Cell. Biol., New Ser. 1985, 22(Cell. Mol. Biol. Plant Stress), 435-46	1-8
A	Chemical Abstracts, volume 104, 1986 (Columbus, Ohio, US), G.P. Lomonossoff et al.: "Structure and in vitro assembly of tobacco mosaic virus", see page 299, abstract 31414h, Mol. Plant Virol. 1985, 1, 43-83	1-8
A	Chemical Abstracts, volume 103, no. 9, September 1985, (Columbus, Ohio, US), see page 181, abstract 66069f, & JP, A, 6041486 (KIRIN BREWERY CO., LTD) 5 March 1985 ./..	1-8

International Application No. PCT/GB 87/00249

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 105, 1986, (Columbus, Ohio, US), D.L. Nuss: "Engineering a plant RNA virus for expression of foreign genetic sequences", see page 165, abstract 147138w, BioEssays 1986, 4(3), 133-4 --	1-8
A	Chemical Abstracts, volume 87, 1977, (Columbus, Ohio, US), D. Zimmern et al.: "The isolation of tobacco mosaic virus RNA fragments containing the origin for viral assembly", see pages 246-247, abstract 129216a, Cell (Cambridge, Mass.) 1977, 11(3), 455-62 cited in the application --	1-8
A	Chemical Abstracts, volume 103, 1985, (Columbus, Ohio, US), T. Wilson et al.: "Nucleocapsid disassembly and early gene expression by positive-strand RNA viruses", see page 307, abstract 19483b, J. Gen. Virol. 1985, 66(6), 1201-7 --	1-8
A	Nature, volume 219, 17 August 1968, S. Rogers et al.: "Use of viruses as carriers of added genetic information", pages 749-751 see the whole document	1-8

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 87/00249 (SA 16846)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The m mbers are as contained in the European Patent Office EDP file on 07/08/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0067553	22/12/82	JP-A-	58051894	26/03/83
		CA-A-	1192510	27/08/85
EP-A- 0153154	28/08/85	AU-A-	3869085	22/08/85
		JP-A-	61005779	11/01/86
EP-A- 0194809	17/09/86	AU-A-	5437886	11/09/86
		JP-A-	62029984	07/02/87

For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82